Virology 413 (2011) 60–71

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Activation of the TGF-β/Smad signaling pathway in oncogenic transformation by v-Rel

Richa Tiwari [1], William Bargmann, Henry R. Bose Jr. *

Section of Molecular Genetics and Microbiology and the Institute of Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712-1095, USA

ARTICLE INFO

Article history:
Received 8 September 2010
Returned to author for revision
11 October 2010
Accepted 1 February 2011
Available online 22 February 2011

Keywords:
TGF-β
Smad3
v-Rel
Rel/NF-κB
Oncogenesis
Transformation

ABSTRACT

v-rel, encoded by the avian reticuloendotheliosis virus, is an acutely transforming member of the Rel/NF-κB family of transcription factors. Transformation by v-Rel is mediated by the aberrant expression of genes that are normally regulated by Rel/NF-κB. Here, we demonstrate activation of the TGF-β/Smad signaling pathway in Rel transformation. RNA and protein levels of key TGF-β and Smad family members (TGF-β2, -β3, TGF-β type II receptor, and Smad3) are upregulated in v-Rel transformed cells with little to no change in c-Rel-expressing cells. Treatment of v-Rel transformed lymphoid cells with kinase inhibitors of the TGF-β receptor dramatically reduces soft agar colony formation whereas addition of TGF-β2 further promotes transformation. Moreover, Smad3 but not Smad2, is selectively activated as the downstream mediator of TGF-β signaling. Blocking Smad3 expression or activity inhibits the oncogenic potential of v-Rel. Overall, TGF-β/Smad signaling is activated at multiple levels and is required for the transforming ability of v-Rel.

Introduction

The Rel/NF-κB family of transcription factors plays important roles in immune and inflammatory responses, proliferation, and apoptosis (Aggarwal, 2004; Bonizzi and Karin, 2004). Rel/NF-κB complexes regulate the expression of a wide range of target genes through specific decameric DNA sites (κB sites) (Chen and Ghosh, 1999). Elevated expression of Rel/NF-κB proteins and/or disruption in their regulation has been implicated in hematopoietic cancers and solid tumors (Karin et al., 2002; Luque and Gelinas, 1997). The earliest evidence of a role for Rel/NF-κB in oncogenic cell transformation came from studies with v-Rel. v-Rel is derived from the transduction of turkey *c-rel* sequences into the avian reticuloendotheliosis-associated retrovirus (REV-A) (Gilmore, 1999). While *c-rel* is weakly oncogenic, *v-rel* is the most efficiently transforming member of the Rel/NF-κB family of transcription factors. Having acquired multiple mutations, v-Rel exhibits altered DNA binding specificity and transactivation potential and is able to escape negative regulation (Hrdlickova et al., 1995; Kabrun et al., 1991; Sachdev and Hannink, 1998). Viruses expressing v-Rel induce avian and mammalian lymphoid cell tumors and, in culture, can immortalize primary splenic lymphocytes (Liss and Bose, 2008). v-Rel also transforms chicken embryonic fibroblasts *in vitro* and induces sarcomas in experimentally infected chickens. Transformation by v-Rel occurs through the inappropriate activation or repression of genes which are normally regulated by Rel/NF-κB family members (Gilmore, 1999). In this report, we have identified specific members of the TGF-β/Smad pathway which are upregulated in v-Rel transformed cells and have found their activation to be critical to the v-Rel transformation process.

The transforming growth factor-β (TGF-β) proteins are members of a large family of cytokines that regulate pivotal biological functions, including cell growth, differentiation, apoptosis, and development (Rahimi and Leof, 2007; ten Dijke and Hill, 2004). Signaling by TGF-β is transmitted to the cell interior through TGF-β receptors. Stimulated by ligand binding, the TGF-β type II receptor (TβIIR) forms a heteromeric complex with the TGF-β type I receptor (TβIR) and phosphorylates the TβIR. Phosphorylation of TβIR relieves its autoinhibition and activates its catalytic kinase domain. This allows TβIR to then phosphorylate Smad2 and Smad3, the canonical TGF-β effector molecules (Abdollah et al., 1997; Macias-Silva et al., 1996). Smad2 and Smad3 phosphorylation triggers their activation, enabling these Smad proteins to form heteromeric complexes with the common mediator Smad4 protein (Correia et al., 2001; Kawabata et al., 1998). These complexes translocate to the nucleus to regulate gene expression in a cell type-specific and ligand dose-dependent manner.

TGF-β signaling has received growing attention in recent years due to its complex role in different cancers, including breast, lung, colon, and skin cancer, as well as cancers of hematopoietic origin (de Jonge et al.,

* Corresponding author at: Mailing address: Department of Molecular Genetics and Microbiology, The University of Texas at Austin, 1 University Station A5000, Austin, TX 78712-1095, USA. Fax: +1 512 471-2130.
E-mail address: bose@mail.utexas.edu (H.R. Bose).
[1] Current address: Ogilvy CommonHealth Medical Education, 402 Interpace Parkway, Parsippany, NJ 07054, USA.

0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.02.002

1997; Go et al., 1999; Inman and Allday, 2000; Ko et al., 1998; Koli et al., 1997; Lange et al., 1999; Takaku et al., 1999; Xu et al., 2000). TGF-β signaling in the early stages of oncogenesis has tumor suppressive effects characterized by antiproliferative activity and induction of apoptosis. In the later stages, cancer cells gain advantage by selective inactivation of tumor suppressor activities of TGF-β while retaining the tumor promoting activities (Massague, 2008). Ultimately, whether TGF-β signaling incurs an oncogenic or tumor suppressive outcome depends on its context-specific effects on cellular targets.

TGF-β/Smad signaling frequently promotes cancers in cooperation with other transcription factors or through crosstalk with other signaling pathways (Derynck and Zhang, 2003). While numerous studies have analyzed how TGF-β/Smads influence epithelial cell growth and cancer, less is known about their role in malignancies of lymphoid origin. Here, we demonstrate the role of TGF-β/Smad signaling in Rel/NF-κB-mediated transformation, which has not been previously addressed. Our studies reveal that unlike c-Rel, v-Rel efficiently induces the mRNA and protein expression of TGF-β and Smad family members, specifically TGF-β2, TGF-β3, TβIIR, and Smad3. Furthermore, activation of TGF-β signaling and Smad3, but not Smad2, is crucial for the transforming ability of v-Rel. Our results demonstrate that v-Rel activates the TGF-β pathway at multiple levels to achieve its oncogenic potential.

Results

v-Rel alters the expression of TGF-β and Smad family members

We recently employed microarray technology to identify global changes in gene expression in v-Rel transformed chicken embryonic fibroblasts (CEFs) and DT40 cells, a chicken B cell line (manuscript in preparation). Results from the microarrays identified four genes from the TGF-β/Smad pathway with at least a 1.6-fold increase in expression in v-Rel transformed cells. These genes were *tgf-β2*, *tgf-β3*, TGF-β type II receptor (*tgfbr2*), and *smad3*. To corroborate these results, Northern blot analysis was carried out to evaluate the ability of v-Rel to alter the expression of all three TGF-β isoforms (*tgf-β1–3*), the TGF-β receptors, and *smad2*, *3*, and *4*. Total RNA was harvested from CEFs and DT40 cultures infected with REV-based retroviruses expressing c-Rel (REV-C) or v-Rel (REV-TW) or with the helper virus CSV as a control. Consistent with our microarray results, the expression of *tgf-β2*, *tgf-β3*, *tgfbr2*, and *smad3* was elevated in v-Rel transformed CEFs and DT40 cells (Fig. 1A). While the levels of *smad4* were increased in v-Rel transformed DT40 cells, there was little change observed in CEFs. The altered expression of members of this pathway was selective as the levels of *tgf-β1*, TGF-β type I receptor (*tgfbr1*), and *smad2* were not significantly altered in either fibroblast or lymphoid cell types. The overexpression of c-Rel also resulted in elevated levels of *tgf-β2* (DT40), *tgf-β3* (DT40), *tgfbr2* (CEFs and DT40), and *smad3* (DT40). However, these changes were less dramatic than those in cells expressing v-Rel. Real-time PCR analysis was also performed using cDNA prepared from RNA infected with the retroviruses described above to quantify the expression of select genes upregulated in the TGF-β/Smad signaling pathway (*tgf-β2*, *smad3*, and *smad2*). In agreement with the Northern blot results, *tgf-β2* and *smad3* expression was dramatically increased in both CEFs and DT40 cells expressing v-Rel relative to control (Fig. 1B). By contrast, *smad2* expression was not significantly changed in v-Rel-transformed cells compared to CSV control. These results demonstrate the ability of v-Rel to effectively induce the expression of specific members of the TGF-β signaling cascade in different cell types.

A temperature-sensitive (ts) v-Rel cell line was used to evaluate whether TGF-β2 and the Smad proteins might represent direct v-Rel targets. The ts v-Rel mutant has impaired DNA binding activity at the non-permissive temperature, resulting in a rapid loss of the transformed phenotype (White and Gilmore, 1993). Total RNA was isolated from separate cultures of cells grown at the permissive temperature (36 °C) and from those shifted to the non-permissive temperature (41 °C) at zero, two, and six hour time points. Northern blot analysis revealed that while *smad3* expression was not significantly changed at the permissive temperature, its expression was dramatically reduced as early as 2 h after the shift to the non-permissive temperature (Fig. 1C). The expression of tgf-*β2* was also downregulated upon shift to the non-permissive temperature, although less dramatically compared to *smad3*. The expression of *smad2* remained unaltered at the non-permissive temperature. These results suggest that Smad3 may be a direct target of v-Rel.

To determine whether increased expression of *smad3* is specific to the v-Rel transformation pathway, RNA levels of *smad3* were also analyzed in a panel of lymphoid cell lines transformed by v-Rel and other avian retroviruses (Fig. S1). Northern blot analysis revealed that expression of *smad3* was strongly elevated (from 2.5 to 6.7 fold) in v-Rel-transformed cells compared to those transformed by other oncogenes, which either failed to upregulate *smad3* expression or did so less effectively (1.8 fold). These results suggest that *smad3* is consistently upregulated and highly expressed in v-Rel-transformed cells relative to cell lines transformed by other oncogenes.

The total protein levels of TGF-β and Smad family members which demonstrated upregulated gene expression were also determined. ELISAs were performed to analyze the levels of active TGF-β2 ligand expressed in v-Rel transformed cells. CEFs and DT40 cells were infected with retroviruses described above, and culture media was harvested over the course of 10–14 days following infection. Consistent with the Northern blot results, v-Rel-transformed cells secreted significantly higher levels of TGF-β2 than control cells (Fig. 2A). In a representative result from one of three experiments, DT40 cells expressing v-Rel produced approximately 1.7–2.6 fold more TGF-β2 than control cells (Fig. 2A, left panel). In v-Rel transformed CEFs, the levels of TGF-β2 produced in the initial three days after infection were three-fold greater than control cells (Fig. 2A, right panel). Over later time points, a 9–16 fold increase in TGF-β2 levels was observed as CEFs expressing v-Rel became morphologically transformed. c-Rel induced a 2.5–3.5 fold increase (CEFs) or minimal change (DT40) in TGF-β2 compared to control cells.

To analyze the protein levels of the remaining TGF-β signaling components with upregulated RNA expression, Western blot analysis was performed on whole cell lysates isolated from the infected CEFs and DT40 cells described above. v-Rel expression increased the levels of the TGF-β type II receptor protein (TβIIR) and Smad3 in both CEFs and DT40 cells (Fig. 2B). Since the TGF-β-dependent activation of Smad3 occurs by the phosphorylation of Ser423 and Ser425 (Attisano and Wrana, 1998), we evaluated whether the phosphorylation status of these sites was affected in v-Rel transformed cells. Consistent with the elevated expression of TβIIR and TGF-β2, the levels of phosphorylated Smad3 were found to be higher in v-Rel-expressing CEFs and DT40 cells. Meanwhile, total Smad2 levels remained unaltered in both cell types, corresponding to its RNA levels. The levels of phosphorylated Smad2 were, however, upregulated in DT40 cells and CEFs. This indicated that, although v-Rel transformed cells do not upregulate total Smad2 RNA and proteins levels, TGF-β2 signaling in these cells is able to activate Smad2. The overexpression of c-Rel modestly raised the levels of TβIIR and total and phospho-Smad3, revealing differences in the ability of v-Rel and c-Rel to upregulate the TGF-β/Smad pathway. These results indicate that v-Rel selectively increases the RNA and protein levels of key TGF-β signaling components relative to c-Rel, which is weakly oncogenic.

The TGF-β signaling pathway increases Smad3 activation in v-Rel transformed cells

Key components of the TGF-β pathway have been shown to be inactivated in immortalized or transformed cell lines to achieve oncogenic potential (de Caestecker et al., 2000; de Jonge et al., 1997;

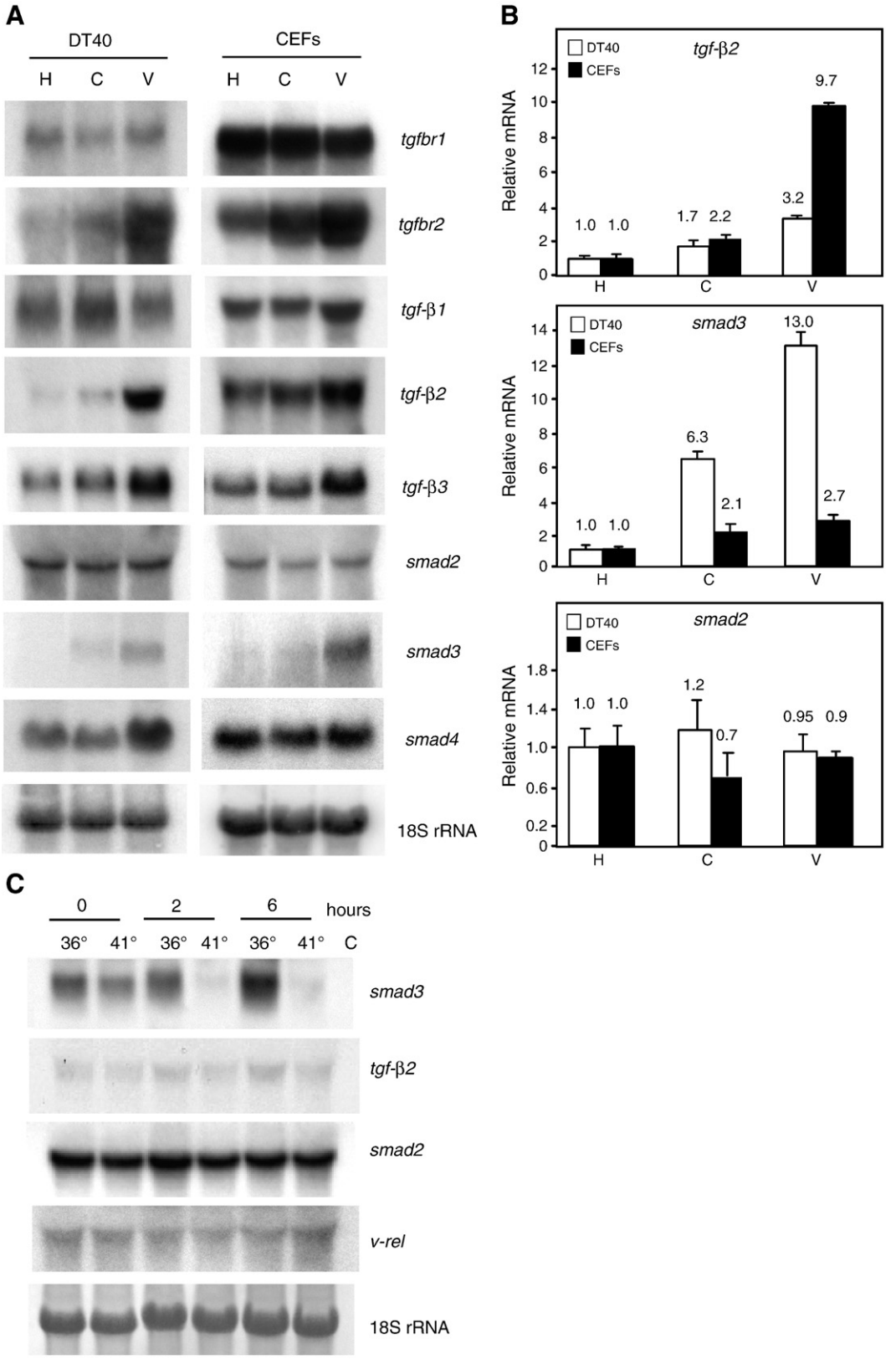

Fig. 1. Expression of mRNAs encoding TGF-β and Smad family members in Rel transformed cells. (A) DT40 cells and CEFs were infected with CSV helper virus (H), or viruses expressing c-Rel (C) or v-Rel (V). Total RNA (10 μg) was harvested for Northern blot analysis to determine mRNA expression of key components of the TGF-β/Smad signaling pathway. (B) Real-time PCR was performed to analyze relative mRNA levels of *smad2*, *smad3*, and *tgf-β2* using cDNA synthesized from DT40 cells and CEFs infected with viruses described in (A). The mRNA expression for each gene was normalized to GAPDH values (C_t), and the extent of upregulation of each gene was compared against the CSV control samples ($\Delta\Delta C_t$). (C) Cultures of a temperature-sensitive v-Rel cell line were grown in parallel at 36 °C and at 41 °C for 0, 2, and 6 h after the shift to the non-permissive temperature. Total RNA (10 μg) harvested from cells was subjected to Northern blot analysis to examine *smad2, smad3,* and *tgf-β2* levels. As an internal control, *v-rel* expression is shown to be unaltered at both the permissive and non-permissive temperatures. For both (A) and (C), hybridization of an oligonucleotide to the 18S ribosomal RNA is shown as a loading control.

Hahn et al., 1996). To determine whether the TGF-β ligand–receptor complexes are functional and can activate their downstream effectors in v-Rel transformed cells, the effect of stimulating and inhibiting the TGF-β pathway in CEFs infected with retroviruses expressing v-Rel or with CSV control virus was evaluated. Cells were treated for 30 min with recombinant TGF-β2 ligand only, treated for 6 h with the SB-431542 inhibitor of the TGF-β type I receptor (TβIR) followed by a 30 min treatment with TGF-β2, or left untreated. To determine whether TGF-β receptor activity was stimulated by ligand treatment or blocked by kinase inhibitor treatment, the phosphorylated levels of the downstream target, Smad3, were analyzed by Western blot. In both v-Rel-expressing and control cells, TGF-β2 treatment resulted in increased levels of phosphorylated Smad3 relative to untreated cells whereas inhibitor treatment dramatically decreased TGF-β2-mediated phosphorylation of Smad3 (Fig. 3A). These effects were most notable in cells transformed by v-Rel, possibly due to the higher levels of TβIIR present on their surface (Fig. 2B). v-Rel transformed cells also activate Smad3 more rapidly compared to control cells, as demonstrated in dose-dependent TGF-β2 treatments (Fig. S2A). Together, these results suggest that the TGF-β pathway is functional and can be effectively stimulated by its ligand or blocked by a kinase inhibitor in v-Rel transformed cells. Reporter assays were also performed in CEFs infected with retroviruses expressing v-Rel and CSV to compare the relative activity of the TGF-β pathway and its downstream activation of Smad3. v-Rel efficiently induced the activity of luciferase reporter constructs containing tandem Smad3-binding sites by at least two-fold over CSV control cells (Fig. 3B). Furthermore, upon TGF-β2 treatment, both control and v-Rel transformed cells revealed increased luciferase activity compared to carrier-treated samples. Consistent with the results of Fig. 3A, ligand treatment led to a slightly greater transactivation of the reporter construct in v-Rel transformed cells relative to control cells. These results indicate that v-Rel may directly activate Smad3 and/or activate the TGF-β pathway which can lead to the activation of Smad3.

Inhibiting TGF-β signaling suppresses colony formation in v-Rel transformed cells

To analyze the importance of elevated levels of TGF-β signaling in v-Rel-transformed cells, experiments were performed to examine the effect of blocking TGF-β signaling. Three histologically distinct v-Rel transformed lymphoid cell lines (160/2T cell line, 123/12 B cell line, and 123/6T non-B/non-T cell line) were treated with the SB-431542 TβIR inhibitor or with DMSO carrier followed by preparation of whole

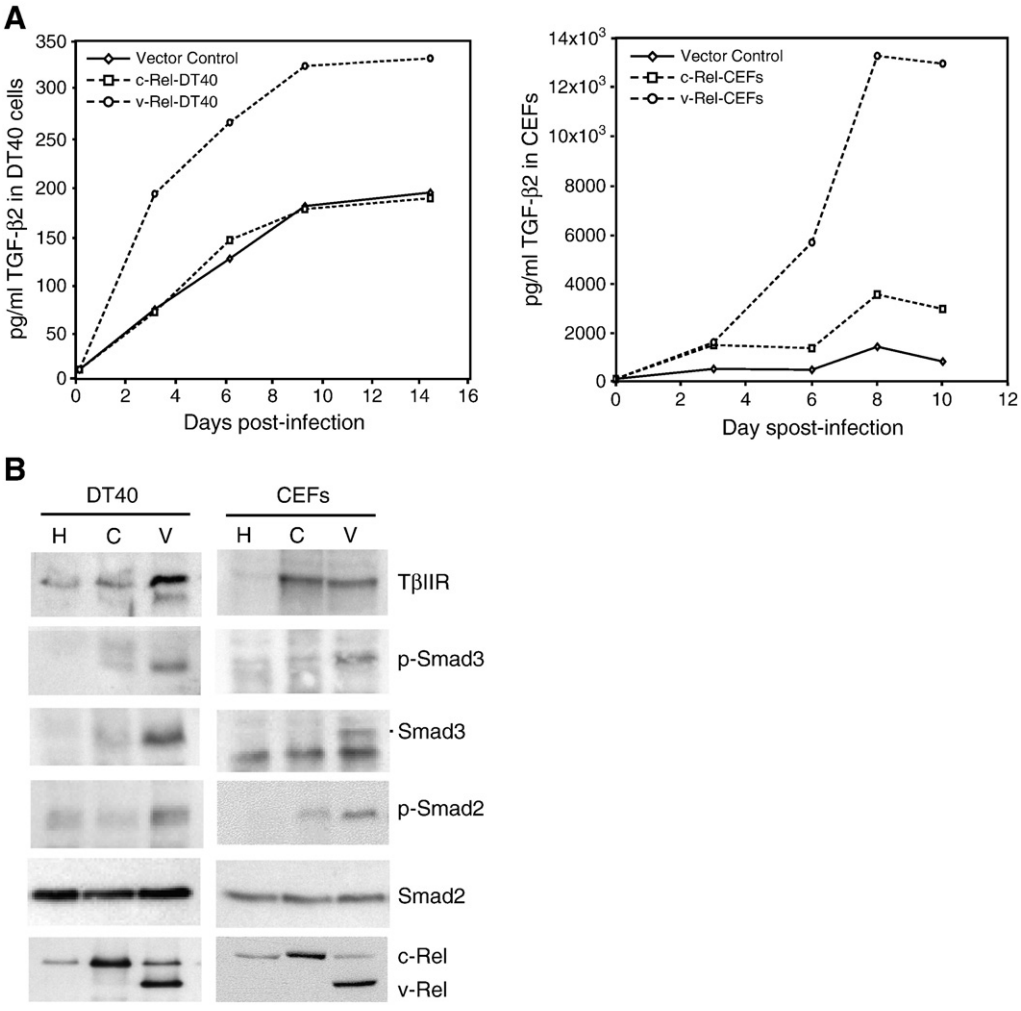

Fig. 2. Increased protein levels of TGF-β/Smad signaling components in v-Rel transformed cells. (A) ELISAs were performed to analyze the levels of TGF-β2 secreted by DT40 cells (left panel) and CEFs (right panel) over a time course of 10–14 days following infection with retroviruses expressing c-Rel, v-Rel, or with CSV helper virus. Media was collected every three–four days from DT40 cells once they reached a density of at least 3×10^6 cells/ml and every two–three days from CEF cultures once they became confluent. Representative results from one of three experiments are shown. (B) Whole cell lysates (40 μg) prepared from DT40 cells and CEFs infected with retroviruses expressing c-Rel (C) or v-Rel (V) or infected with CSV (H) were analyzed by Western blot for the expression of TβIIR, total and phosphorylated Smad2, and total and phosphorylated Smad3.

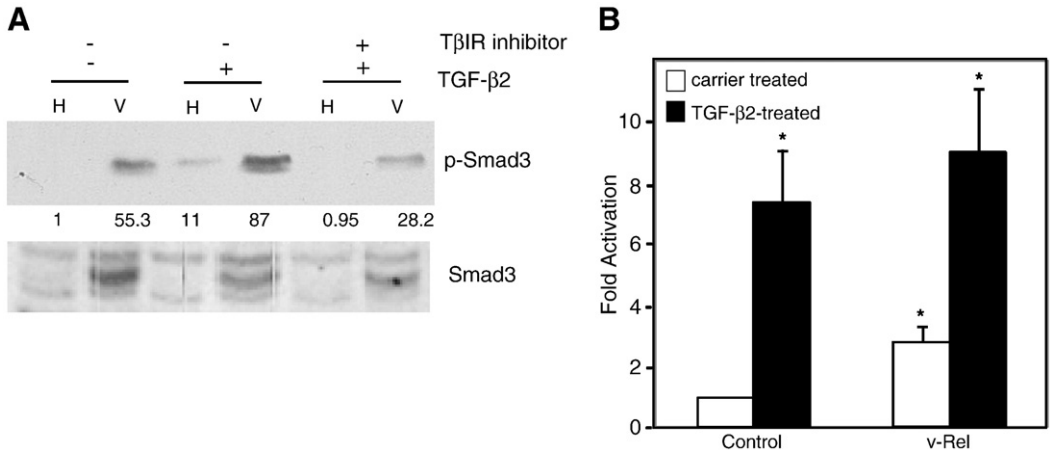

Fig. 3. Activation of Smad3 in v-Rel transformed cells. (A) CEFs infected with viruses expressing v-Rel (V) or with CSV helper virus (H) were left untreated, treated with 2 ng/ml TGF-β2 for 30 min, or treated with 3 μM TβIR inhibitor for 6 h followed by 30 min treatment with 2 ng/ml TGF-β2. Whole cell lysates prepared from these cells were analyzed for the expression of total and phosphorylated Smad3 by Western blot. (B) CEFs infected with retroviruses expressing v-Rel or CSV (control) were transiently transfected with a reporter vector containing tandem Smad3 binding sites. At 32 h post-transfection, lysates were prepared from cells treated with 2 ng/ml TGF-β2 or carrier for the measurement of luciferase activity. The average and standard error of three experiments is shown (*$P<0.05$).

cell lysates and Western blot analysis. While total Smad3 levels remained constant, phospho-Smad3 expression was reduced in all three cell lines treated with the inhibitor compared to the DMSO control (Fig. 4A). Cell counts over a two day time course did not reveal significant differences in growth or cell death between inhibitor-treated and carrier-treated cells for each cell line (data not shown). Uninfected DT40 cells were also treated with the TβIR inhibitor to assess its effect on non-v-Rel transformed cells. Unlike the v-Rel cell lines, uninfected DT40 cells have significantly lower levels of Smad3. Thus, a reduction in phospho-Smad3 levels in inhibitor-treated DT40 cells was observed only after a longer exposure (Fig. S2B). The expression of total and phospho-Smad2 was also analyzed in the three v-Rel transformed cell lines treated with or without the inhibitor. In accordance with results of Fig. 2B where phospho-Smad2 levels are slightly increased in v-Rel transformed cells, inhibitor treatment had the opposite effect of reducing the levels of phospho-Smad2 (Fig. 4A). This is not surprising as Smad2 is also a downstream effector of TGF-β signaling and can be activated by these receptors.

The effect of the SB-431542 TβIR inhibitor on v-Rel transformation was then evaluated. The three different v-Rel transformed cell lines were treated with the inhibitor, plated in soft agar, and scored for colony formation after 10 days. In the T cells (160/2), inhibitor treatment reduced colony growth in soft agar by 97% compared to DMSO carrier (Fig. 4B). With the B cell line (123/12), colony formation was inhibited by almost 50% whereas in the non-B/non-T cells (123/6T), an 80% reduction in colony formation was observed with inhibitor treatment relative to control. Together, these results suggest that blocking the activity of TβIR strongly suppresses the transformed phenotype of v-Rel-expressing cells. The transformation potential of non-v-Rel transformed DT40 cells treated with the TβIR inhibitor was also tested. No significant difference was observed in colony formation in these cells compared to carrier-treated samples (Fig. 4B), suggesting that reduction in colony formation is not a general effect of the inhibitor.

While receptor-activated Smads are the major effectors of TGF-β signaling, other pathways have been shown to be activated in certain contexts and cell types, particularly MAPK pathways (Arsura et al., 2003; Derynck and Zhang, 2003; Javelaud and Mauviel, 2005). Therefore, changes in MAPK signaling were also evaluated in SB-431542 TβIR inhibitor-treated cells. We have previously observed elevated levels of phosphorylated ERK and JNK in v-Rel transformed cells (Kralova et al., 2010). Treatment of v-Rel transformed DT40 cells with the TβIR inhibitor did not reveal differences in the activation of the ERK, JNK, and p38 MAPK signaling pathways (Fig. S2C). These results suggest that the reduction in v-Rel colony formation is mainly dependent on Smad activation.

To corroborate the results of the colony assays performed with the SB-431542 TβIR inhibitor, the effect of blocking TGF-β signaling on v-Rel transformation was also tested using a different TβIR inhibitor, SB-525334 (Grygielko et al., 2005). The 160/2 v-Rel transformed cell line was treated with the SB-525334 inhibitor or DMSO carrier, plated in soft agar, and scored for colony formation. This inhibitor produced approximately an 80% decrease in colony formation, an effect similar to that observed with the SB-431542 inhibitor (Fig. 4C). Inhibitor-treated 160/2 cells revealed a corresponding reduction in the levels of phosphorylated Smad3 with no changes in the levels of total Smad3. Uninfected DT40 cells treated with the same inhibitor did not reveal significant differences in colony numbers when compared to DMSO carrier-treated cells.

TGF-β signaling is important in the initiation of v-Rel transformation

The experiments described above analyzed the role of TGF-β signaling in cells stably transformed by v-Rel. To determine whether TGF-β signaling is involved in the initial stages of v-Rel transformation, primary splenic lymphocytes were analyzed for their ability to form colonies in soft agar after TβIR inhibitor treatment. Primary splenic lymphocytes were harvested from three-week old chickens and infected with retroviruses expressing v-Rel. The next day, cells were treated with 1, 3, and 5 μM of the SB-431542 TβIR inhibitor followed by preparation of whole cell lysates for Western blot analysis. A dose-dependent decrease in the levels of phosphorylated Smad3 was observed with no change in total Smad3 levels (Fig. 5A). Cells were also plated in soft agar after inhibitor treatment and colonies scored 10 days later. Primary splenic lymphocytes infected with the CSV control virus are not transformed, and therefore did not form colonies in soft agar (data not shown). The colony-forming ability of primary splenic lymphocytes expressing v-Rel was reduced with increasing concentrations of the inhibitor (Fig. 5B). While 3 μM inhibitor treatment led to approximately a 40% decrease in colony numbers compared to control DMSO-treated cells, 5 μM inhibitor treatment produced the most dramatic reduction in colony formation (70%).

In complementary experiments, primary splenic lymphocytes infected with retroviruses expressing v-Rel were treated with increasing concentrations of TGF-β2. Whole cell extracts were prepared from these cells, and the expression of total and phospho-Smad3 was analyzed by Western blot. Ligand treatment augmented the levels of phospho-Smad3 in a dose-dependent manner while total Smad3 levels remained constant in all samples (Fig. 5C). Following exposure to increasing concentrations of TGF-β2, primary splenic lymphocytes expressing v-Rel were also plated in soft agar. TGF-β2 treatment at 0.2 ng/ml enhanced colony formation by two-fold compared to carrier-treated cells (Fig. 5D). Interestingly, at higher doses (0.8 ng/ml), TGF-β2 elicited a negative effect on the ability of primary splenic lymphocytes to form colonies in soft agar.

Experiments were also performed to determine whether TGF-β2 can enhance the weak transformation potential of c-Rel. Primary splenic lymphocytes were infected with CSV and the REV C retrovirus, treated with 0.5 ng/ml TGF-β2, and analyzed for transformation potential through liquid transformation assays (since c-Rel transformed cells do not form colonies in soft agar). Treatment with TGF-β2 ligand did not lead to a significant increase in the ability of c-Rel to transform splenic lymphocytes in liquid culture (Fig. S3B). Overall, these experiments indicate that TGF-β signaling is important in the initiation of v-Rel transformation and plays a more important role in the oncogenic potential of v-Rel over that of c-Rel.

Inhibiting the activity or expression of Smad3 reduces the transforming ability of v-Rel

The results above revealed the importance of Smad proteins in relaying TGF-β signaling (Figs. 3 and 4). To gain additional insight into the contribution of elevated levels of Smad proteins, particularly Smad3, in v-Rel transformation, experiments were performed to examine the effects of reducing Smad3 activity. Mutation of the serine residues in the 'SSXS' domain of receptor-activated Smads has been previously described to alter their ability to be phosphorylated by the TβIR (Kawabata et al., 1999; Liu et al., 1997). DN Smad3 was generated by mutating the serine residues to alanine residues, resulting in a mutant that cannot be activated by TβIR.

To determine the effect of DN Smad3 on Smad3-dependent transcription, luciferase reporter assays were performed. A pGL3 reporter construct containing tandem Smad3-binding sites from the PAI-1 promoter was cotransfected into CEFs with expression plasmids (Rc/RSV) carrying wild-type (WT) Smad3 or DN Smad3. Cells transfected with WT Smad3 induced luciferase activity by 10-fold compared to cells transfected with the empty Rc/RSV vector (Fig. S4A). Interestingly, DN Smad3 also induced luciferase activity that surpassed the basal level of activity observed with empty Rc/RSV vector. This result is consistent with the observation in certain contexts that overexpression of dominant negative Smad proteins can lead to the activation of luciferase activity in

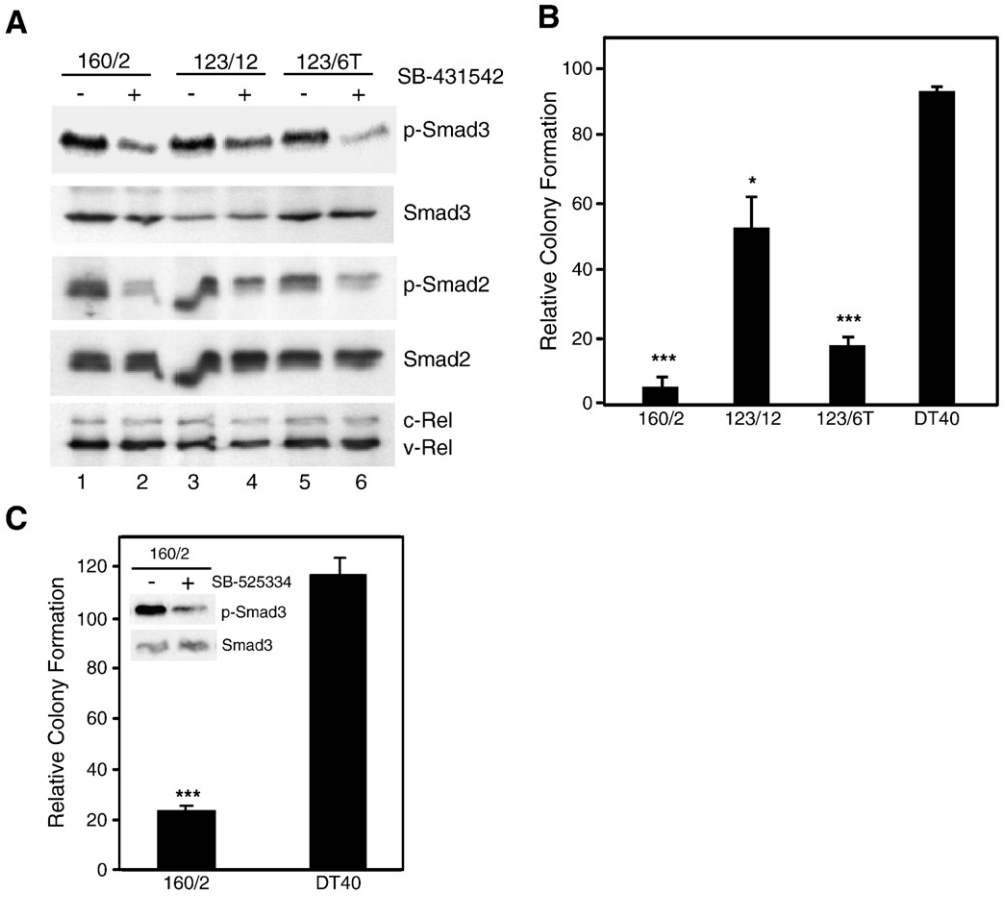

Fig. 4. Effects of inhibiting TGF-β signaling activity in v-Rel transformed cells. (A) Three histologically distinct v-Rel transformed cell lines (160/2, 123/12, 123/6T) were treated for 6 h with 3 μM SB-431542 TβIR inhibitor (lanes 2, 4, and 6) or carrier DMSO (lanes 1, 3, and 5) as control. Whole cell lysates were prepared from these cells and analyzed by Western blot for the expression of total and phosphorylated Smad3, total and phosphorylated Smad2, and Rel proteins. (B) The v-Rel transformed cell lines described in (A) and uninfected DT40 cells were plated in soft agar and scored for colony formation after 7–10 days. The number of colonies formed by DMSO-treated samples was standardized to 100 for each cell line. The number of colonies formed by the SB-431542 TβIR inhibitor-treated cells was normalized based on this standard and represented here. (C) 160/2 and uninfected DT40 cells were treated with 3 μM SB-525334 TβIR inhibitor or DMSO carrier for 6 h before plating in soft agar. Whole cell lysates were also prepared from the treated 160/2 cells and analyzed for the expression of total and phosporylated Smad3 by Western blot, shown in the inset. The number of colonies formed by the inhibitor-treated cells was normalized against the DMSO-treated samples for each cell type and is represented here. For (B) and (C), the average and standard error of three experiments is shown (*$P<0.05$, ***$P<0.001$).

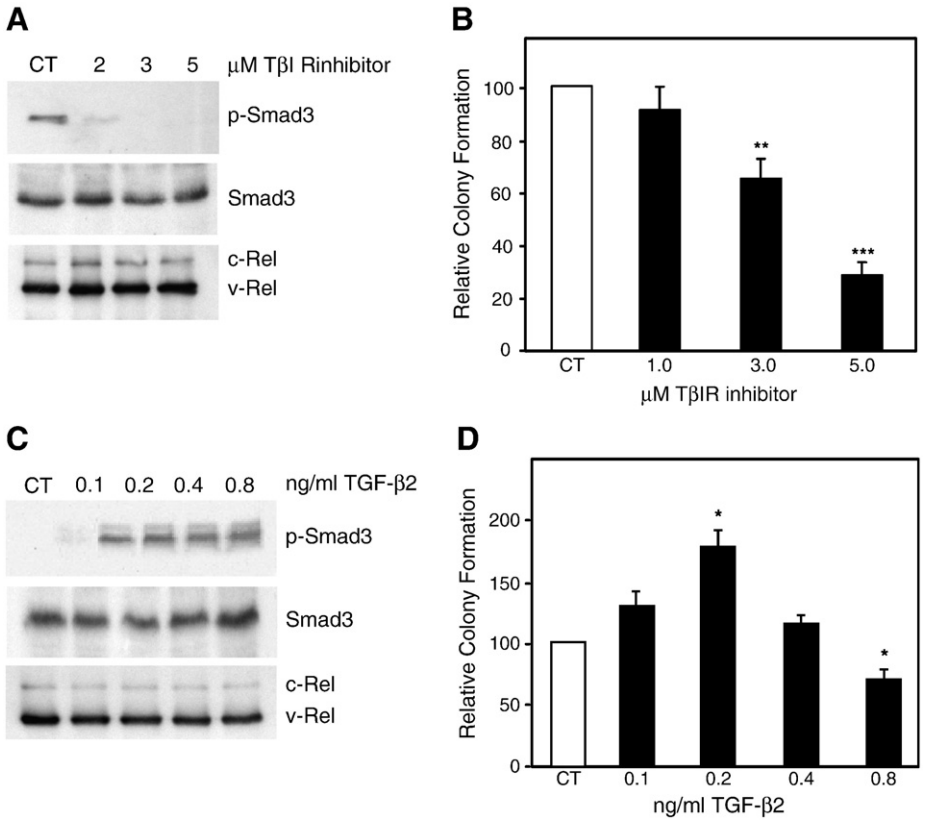

Fig. 5. TGF-β signaling in transformation of primary splenic lymphocytes by v-Rel. (A) REV-based retroviruses expressing v-Rel (REV-TW) were used to infect primary splenic lymphocytes from three-week old chickens. The infected cells were treated with 1, 3, or 5 μM SB-431542 TβIR inhibitor or DMSO carrier (CT) for 6 h and then harvested for Western blot to analyze expression of phosphorylated and total Smad3, and Rel proteins. A slightly longer exposure of the phospho-Smad3 blot is shown to demonstrate the dose-dependent decrease in phosphorylated Smad3 levels. (B) At 24 h after infection with REV-TW, primary splenic lymphocytes were pretreated and plated in soft agar with the indicated doses of TβIR inhibitor. Colonies were scored after 10 days. (C) Primary splenic lymphocytes infected with the REV-TW retrovirus were treated with 0.1, 0.2, 0.4, or 0.8 ng/ml TGF-β2 or carrier (CT) for 30 min. Whole cell lysates were then prepared and analyzed by Western blot for the expression of phosphorylated and total Smad3, and Rel proteins. (D) Cells were pretreated and plated in soft agar with the indicated doses of TGF-β, and colonies were scored after 10 days. For both (B) and (D), colony numbers were normalized to those derived from carrier-treated cells. The average and standard error of three experiments is shown (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

the absence of receptor activation (Poncelet et al., 1999; Wu et al., 1997; Zhang et al., 1996). Upon TGF-β2 treatment, cells transfected with the empty Rc/RSV expression vector demonstrated an almost seven-fold increase in luciferase activity, suggesting a functional endogenous Smad transcriptional response (Fig. S4A). WT Smad3 also exhibited a robust response to TGF-β2 whereas DN Smad3 exhibited little to no response relative to carrier-treated samples. Western blot analysis of whole cell lysates prepared from cells used in the reporter assays confirmed the appropriate expression of WT and DN Smad3 (Fig. S4B). The levels of phospho-Smad3 were equivalent among all samples treated with the carrier solution. In response to TGF-β2, phospho-Smad3 levels increased in cells transfected with empty Rc/RSV vector and did so even more dramatically in WT Smad3-expressing cells. TGF-β2 exposure failed to significantly induce Smad3 phosphorylation in DN Smad3-expressing cells over that of carrier-treated samples.

Once the appropriate activity of the Smad3 mutants was confirmed, experiments were performed to assess the effects of their overexpression in v-Rel transformed cells. RSV-based retroviral vectors (DS) constructed to express WT or DN Smad3 were used to infect v-Rel transformed cell lines. Whole cell lysates were prepared from these infected cells and analyzed by Western blot. In all three cell lines, WT and DN Smad3 were overexpressed above the endogenous Smad3 levels observed in the DS vector control samples (Fig. 6A). Cells overexpressing WT Smad3 had higher levels of phosphorylated Smad3 compared to DS control. Phospho-Smad3 expression was decreased in cells overexpressing DN Smad3 compared to those overexpressing WT Smad3, indicating the reduced ability of the dominant negative mutant to be activated by the TGF-β receptor.

The Smad3-encoding viruses were then evaluated for their ability to alter the transformed phenotype of the v-Rel transformed cell lines. Cells were plated in soft agar after infection with the retroviruses and then scored for colony formation after 10 days. The overexpression of WT Smad3 promoted colony formation by almost two fold compared to DS empty vector control in 160/2 cells (Fig. 6B). While this effect was not as dramatic in 123/12 and 123/6T cells, ectopic expression of WT Smad3 still produced a 1.5-fold increase ($P<0.05$) in colony formation over that of control samples. In contrast, all three cell lines infected with retroviruses expressing DN Smad3 exhibited approximately a 50% reduction in colony numbers.

In complementary experiments, the effect of reducing endogenous levels of Smad3 on v-Rel transformation was analyzed. Retroviral vectors were constructed to express shRNAs that targeted *smad3* as well as *smad2,* to determine the extent of its involvement in v-Rel transformation. The ability of a specific shRNA to effectively reduce the levels of its endogenous target was evaluated in 160/2 cells. Cells were infected with empty viruses (RCAS), viruses encoding shRNAs specific for *smad3* and *smad2*, or an shRNA specific for luciferase (luc) as a negative control. The expression of Smad3 and Smad2 was reduced by 20–40% in cells expressing the specific shRNAs relative to cells infected with the control RCAS and luc shRNAs (Fig. 6C). Luciferase assays of CEFs infected with shRNAs specific to *smad3* were also performed using a Smad3-specific reporter vector. The results demonstrated approximately a 50% reduction in luciferase activity in Smad3 shRNA-infected cells compared to those infected with the RCAS control (Fig. 6D),

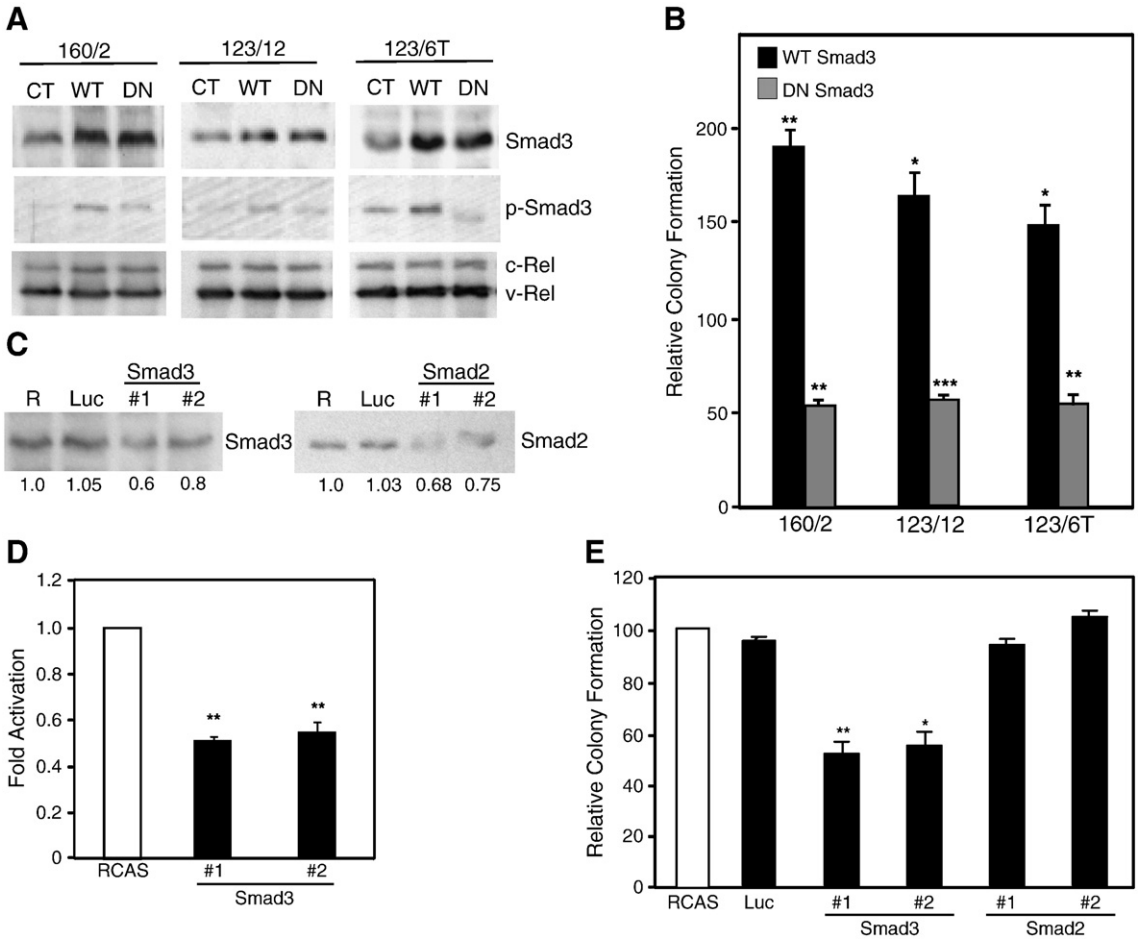

Fig. 6. Effect of altered Smad3 activity and expression on v-Rel transformation. (A) v-Rel transformed cell lines (160/2, 123/12, and 123/6T) were infected with control DS virus (CT) or with one expressing WT or DN Smad3. Whole cell lysates were prepared from these cells and analyzed by Western blot for the ectopic expression of Smad3, phosphorylated Smad3, and Rel proteins. (B) The v-Rel transformed cell lines described in (A) were plated in soft agar eight days post-infection and scored for colony formation after 7–10 days. The number of colonies formed by control DS-infected cells was standardized to 100 for each cell line. The number of colonies formed by cells infected with retroviruses expressing WT or DN Smad3 was normalized based on this standard. The average and standard error of three experiments is shown (*P<0.5, **P<0.01, ***P<0.001). (C) Empty RCAS retroviruses (R) or RCAS viruses expressing an shRNA specific to luciferase (Luc), *smad3*, or *smad2* were used to infect 160/2 cells. Whole cell lysates from infected cells were harvested eight days after infection and analyzed by Western blot for the expression of Smad3 or Smad2. The extent of upregulation of each Smad protein was quantitated and is indicated below the corresponding Western blot. (D) Luciferase reporter assays were performed in CEFs about 7–8 days post-infection with the *smad3* shRNAs. Cells were transiently transfected with a Smad-responsive reporter and treated with TGF-β2 about 30 min prior to harvest. At 32 h post-transfection, lysates were prepared from cells for the measurement of luciferase activity. Fold activation was calculated in reference to baseline activity of cells infected with the empty RCAS retroviral vector. The average and standard error of three experiments is shown (**P<0.01). (E) The RCAS-infected 160/2 cells described above were also plated in soft agar and scored for colony formation after eight days. The number of colonies from each experiment was normalized to control RCAS-infected cells. The average and standard error of four experiments is shown (*P<0.5, **P<0.01).

indicating the extent to which these shRNA constructs can block Smad3 expression and signaling.

The shRNA-encoding retroviruses were then evaluated for their ability to alter the transformed phenotype of 160/2 cells. Cells infected with viruses encoding the luc shRNA had no effect on colony formation. Viruses encoding shRNAs specific for *smad3* reduced the ability of these cells to form colonies in soft agar by approximately 50% relative to cells infected with empty control virus (Fig. 6E), correlating with the effect of DN Smad3 on the transforming ability of v-Rel. In agreement with the RNA and protein expression analyses, shRNAs against *smad2* did not lead to significant effects on the transformation potential of v-Rel. Taken together, these colony formation assays indicate that TGF-β-activated Smad3 is an important mediator of the v-Rel transformation process.

Discussion

Certain oncogenes have been found to activate TGF-β signaling to obtain an advantage over normal cells for tumor growth and invasion (Janda et al., 2002; Muraoka et al., 2003; Wang et al., 2010). Here, we report that v-Rel activates key components of the TGF-β/Smad signaling pathway, which contributes to its oncogenicity. The expression of *tgf-β2*, *tgf-β3*, *tgfbr2*, and *smad3* RNA was increased in v-Rel transformed fibroblasts and lymphoid cells relative to control cells (Fig. 1). The protein levels of TGF-β2, TβIIR, and the total and phosphorylated levels of Smad3 were also elevated in these cells (Fig. 2). Furthermore, TGF-β2 and activation of Smad3 were shown to be important for v-Rel colony formation. In comparison to v-Rel, the weakly oncogenic c-Rel had only a small to moderate effect on the expression and activity of TGF-β and Smad signaling components and did not require the activation of this pathway for its ability to induce transformation. Overall, differences in the expression of TGF-β and Smad family members between cells expressing c-Rel and v-Rel may contribute to the enhanced oncogenicity of v-Rel relative to c-Rel.

v-Rel transformed cells are unique in their requirement for the activation of TGF-β signaling for maintaining their transformed phenotype. Unlike DT40 cells, v-Rel transformed cell lines exposed to the TβIR inhibitors demonstrated a dramatic reduction in colony formation (Fig. 4). These results are consistent with previous reports that have demonstrated impaired growth of various tumor cells

treated with the SB-431542 inhibitor (Halder et al., 2005; Hjelmeland et al., 2004; Matsuyama et al., 2003). Human osteosarcoma, malignant glioma, human breast cancer, pancreatic adenocarcinoma, and colon cancer cells have been shown to be sensitive to the effects of this TβIR inhibitor due to its ability to block the tumor-promoting effects of TGF-β. However, genes that contribute to the maintenance of v-Rel transformation may be distinct from those involved in the initial stages of transformation. Likewise, the role of TGF-β signaling may vary depending on the context and stage of transformation. Studies with v-Rel enable the analysis of genes which contribute to the beginning stages of transformation. The exposure of primary splenic lymphocytes expressing v-Rel to the SB-431542 TβIR inhibitor indicated that TGF-β signaling is also important in the initiation of v-Rel transformation (Fig. 5).

In complementary experiments, recombinant TGF-β2 enhanced colony formation in the primary splenocytes expressing v-Rel. However, this effect was dose-dependent as higher TGF-β2 concentrations had a negative impact on v-Rel transformation. A similar inhibitory effect on colony formation was observed when 160/2 v-Rel transformed cells were treated with increasing concentrations of TGF-β2, suggesting that this result is not cell type-specific or transformation stage-dependent (data not shown). This opposing, biphasic effect of TGF-β is not without precedent. For instance, TGF-β2 has been shown to stimulate osteoclast differentiation and proliferation of murine hematopoietic stem cells at low concentrations but block this process at higher doses (Henckaerts et al., 2004; Karst et al., 2004). Vaidya et al. also reported a similar bidirectional effect of TGF-β1 on hematopoietic cell proliferation and attributed this phenomenon to the differential activation of pathways by TGF-β1 (Kale and Vaidya, 2004). Based on our results and those from other studies, it appears that certain pathways may be modulated by subtle changes in TGF-β concentration, which may influence the outcome of TGF-β responses. Precisely which pathways mediate the pro-oncogenic responses of TGF-β in v-Rel transformed cells and which evoke tumor-suppressive effects is not clear.

Many TGF-β-inducible pro-oncogenic pathways function independently of Smads or require cooperation between Smads and other pathways under transforming conditions (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). Signaling by the MAPK pathways was not altered in v-Rel transformed cells treated with the TβIR inhibitor, indicating that this alternative pathway is not activated in response to TGF-β. Although we cannot rule out the possibility that TGF-β activates other pathways in v-Rel transformed cells, Smad3 appears to be the principal effector protein (Figs. 3 and 4). Furthermore, our results reveal the importance of active, phosphorylated Smad3 in mediating TGF-β responses to ultimately support v-Rel transformation. The relatively higher levels of TGF-β2 produced by v-Rel transformed cells are likely to stimulate TGF-β signaling in an autocrine and paracrine manner (Fig. 2). Thus, WT Smad3 can be phosphorylated and continually activated in a physiological manner, leading to enhanced v-Rel transformation potential whereas DN Smad3 is unable to promote colony formation (Fig. 6).

The role of Smad3 in oncogenesis is not clearly defined. Several studies support a tumor suppressive role for Smad3 in cancer. For instance, reduced expression of Smad3 has been detected in human gastric tumors and skin carcinogenesis and has been shown to promote myeloid leukemia (Han et al., 2004; He et al., 2001; Kurokawa et al., 1998; Sood et al., 1999). Our studies suggest a pro-oncogenic role for Smad3, whereby the increased expression and TGF-β-dependent activation of Smad3 promotes v-Rel transformation. Interestingly, while the expression of total Smad2 was unchanged (Figs.1 and 2), increased levels of TGF-β ligand and receptor in v-Rel transformed cells were likely sources of higher levels of phosphorylated Smad2. Despite this, Smad2 did not appear to play an important role in v-Rel transformation (Fig. 6). Other studies have also reported differing and complex roles of the Smad proteins in cancer. For instance, a recent study demonstrated tumor-promoting and metastatic activities of Smad3 in breast cancer whereas Smad2 played a tumor-suppressor role (Petersen et al., 2009). Overall, results from various studies, including ours, demonstrate that Smad2 and Smad3 may be selectively activated and may play different roles in different types of cancer.

Some studies have reported that deficient TGF-β/Smad signaling is important in the progression of head and neck cancers and B-chronic lymphocytic leukemia that are characterized by constitutive Rel/NF-κB activation (Cohen et al., 2009; Zaninoni et al., 2003). However, our results provide evidence for the activation of TGF-β/Smad signaling as an important player in v-Rel transformation. v-Rel has likely employed TGF-β signaling mediated by Smad3 to selectively activate genes that promote the transformation process. Moreover, genes encoding for the adhesion receptor CD44 and integrin β8 are upregulated in v-Rel transformed cells in our microarrays (manuscript in preparation). These genes have also been identified as TGF-β/Smad targets by other groups and are particularly interesting as they are implicated in the pathogenesis of malignancies caused by aberrant Rel/NF-κB activity (Margadant and Sonnenberg, 2010; Robetorye et al., 2002; Tzankov et al., 2003; Verrecchia et al., 2001). Studies to determine whether upregulated expression of such genes is TGF-β/Smad-dependent in v-Rel transformed cells would provide further insight into the importance of TGF-β/Smad signaling in Rel/NF-κB oncogenesis.

Materials and methods

Primary chicken embryonic fibroblasts (CEFs) were prepared from 10 to 11 day-old embryos (Charles River SPAFAS, Wilmington, MA) and grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 5% chicken serum (CS), and 1% Antibiotic–Antimycotic reagent (Invitrogen Life Technologies, Carlsbad, CA) at 37 °C with 8% CO_2. The following avian cell lines used in this study were also maintained in the same conditions: B-cell lines transformed by avian leukosis virus (DT40 and DT95), a T-cell line transformed by Marek's disease virus (RP1), a macrophage-like cell line transformed by avian myeloblastosis virus (BM2), a macrophage cell line transformed by myelocytomatosis virus (HD11), and an erythroid cell line transformed by the avian erythroblastosis virus (AEV). The v-Rel-transformed cell lines include a T-cell line (160/2), a B-cell line (123/12), a non-B/non-T cell line (123/6T) (Hrdlickova et al., 1994), a mixed population lymphocytic cell line (C4-1), and a temperature-sensitive v-Rel cell line (White and Gilmore, 1993).

Cloning and mutagenesis of Smad3

Full length avian Smad3 was amplified by RT-PCR from cDNA derived from chicken brain tissue using the GC-melt cDNA Advantage Kit (BD Clontech Biosciences, Mountain View, CA). The primers were designed from sequences flanking the avian Smad3 coding region and are as follows: Smad3 Forward 5′-GAATTGACGTCGATATCGGCA-GAGCCAGCATGTCCTCCATCCTGC-3′ and Smad3 Reverse 5′-CTTAAGCGGCCGCGATATCCCTTAGGAGACGCTGGAGCA-3′. PCR-amplified Smad3 was then inserted into the pGEM-T easy vector (Promega Corporation, Madison, WI), generating pGEM-WT-Smad3. To generate the dominant negative (DN) Smad3 mutant, point mutations were introduced to sequences in pGEM-WT-Smad3 encoding the SSXS domain in the C-terminal region of the protein using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers designed were as follows: 5′-GAGCATCCGCTGCgccgcaGTCgcaTAAGG-GATATCGC-3′. Lower case letters indicate position of the mutagenic nucleotides. The sequences of the PCR-amplified wild-type (WT) and mutagenized DN *smad3* were confirmed by the DNA Sequencing Facility at the University of Texas at Austin.

Retroviral vectors and preparation of virus stocks

Three different retroviral vector systems were employed in these studies. The REV-based retroviral vectors, pREV-C and pREV-TW, contain

the coding region of c-Rel and v-Rel, respectively (Nehyba et al., 1997). The DS-based retroviruses were constructed by cloning wild-type and DN *smad3* into the pTZDS-XB vector (containing the 3′ portion of *env* and one LTR) within the *Xho*I/*Bss*HII sites (Hrdlickova et al., 2001). The Rous sarcoma virus (RSV)-based shRNA vector system is composed of pRFPRNAiC and RCASARNAi (Das et al., 2006). Gene specific oligonucleotides were first cloned into pRFPRNAiC. A *Not*I–*Cla*I restriction fragment containing this cassette downstream of the chicken U6 promoter was excised and cloned into RCASARNAi to generate a retroviral vector expressing each shRNA. The oligos encoding the shRNA hairpins for each gene are described in Supplementary Materials and Methods.

Retroviral stock preparation has been previously described (Liss and Bose, 2002). Briefly, primary CEFs were plated at a density of 6×10^5 cells/60 mm tissue culture plate 24 h prior to transfection. For generation of REV-based retroviral stocks, 10 μg of pREV retroviral DNA together with 0.3 μg of pCSV11S3 (encoding the chicken syncytial helper virus) was mixed with 0.25 M $CaCl_2$ and an equal volume of BES-buffered saline solution. Viral supernatant fluids were harvested 10–14 days after transfection and stored at −80 °C. RCASARNAi retroviral constructs were transfected as described above but without pCSV11S3. DS retroviral stocks were prepared by linearizing pTZDS-XB by digestion with *Sal*I and ligating to the *Sal*I-digested pREP-B vector (containing one LTR, *gag*, *pol*, and the 5′ portion of *env*). The ligated DNAs (4 μg) were then directly used for transfection of CEFs. Retroviral stocks were harvested 6–7 days post-transfection before −80 °C storage.

To quantitate the viral stocks, virus aliquots were added to a Hybond N+ nylon membrane (Amersham Biosciences, Piscataway, NJ) using a dot–blot transfer unit. For controls, stocks of viruses with titers previously determined by immunohistochemical assays were used (Liss and Bose, 2002). Membranes were dried and crosslinked with UV. Pre-hybridization of the membranes was carried out at 55 °C using the UltraHyb solution (Ambion, Austin, TX) after which radiolabeled probe was added (4×10^6 cpm/ml). Probes for the DS viruses were randomly labeled with [^{32}P]dCTP (DECAprime II Kit, Ambion), while those for the REV-based viruses were labeled with [^{32}P]dATP. The hybridized probe was detected the next day using the ImageQuant software, and titers were normalized to known titers of the control virus.

Western blot analysis

Proteins from whole cell lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Optitran nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Equal loading of lysates was analyzed by staining with Ponceau S. Primary antibodies used were as follows: Smad3 (Millipore, Billerica, MA), phosphorylated Smad3 (R&D Systems, Minneapolis, MN), TβIIR (Abcam, Cambridge, MA), Smad2 and phosphorylated Smad2 (Cell Signaling Technology, Boston, MA). A monoclonal antibody HY87 was used for the detection of v-Rel (Hrdlickova et al., 1994). Secondary antibodies employed for this study were horseradish peroxidase-conjugated donkey anti-rabbit IgG, donkey anti-mouse IgG, and donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein expression was visualized by Western lighting chemiluminescence reagent (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA).

Northern blot analysis

Total RNA was prepared from the TriReagent (Ambion), according to the manufacturer's instructions. Northern blots were performed as previously described (Majid et al., 2006). Briefly, 10 μg of each sample was separated by electrophoresis on a 1% agarose-formaldehyde gel. RNA transfer was performed overnight onto a Hybond N+ nylon membrane (Amersham Biosciences). The membrane was then dried, and RNA was crosslinked with UV. Equal loading and transfer of RNA was confirmed by methylene blue staining. Membranes were prehybridized at 55 °C using UltraHyb solution (Ambion) followed by addition of radiolabeled probes (4×10^6 cpm/ml). Random priming with [^{32}P]dCTP was used to label the probes (DECAprime II Kit, Ambion). cDNAs for generating probes were obtained from the BBSRC ChickEST Database and are listed as follows: *tgf-β1* (ChEST673K20), *tgf-β2* (ChEST539M16), *tgf-β3* (ChEST967G20), *tgfbr1* (ChEST507H10), *tgfbr2* (ChEST863E11), *smad2* (ChEST261L19), *smad3* (ChEST768O18), and *smad4* (ChEST747A11).

Real-time PCR

RNA (60 ng) prepared from CEFs and DT40 cells described above was reverse-transcribed into cDNA using the Superscript First Strand Synthesis System (Invitrogen Life Technologies). The synthesized cDNA was combined with Smad2, Smad3, or TGF-β2-specific primers or GAPDH primers as control, TaqMan MGB probes specific for each gene (Applied Biosystems), and FastStart Universal Probe Master Mix (Roche, Indianapolis, IN). PCR reactions were performed using standard 96-well plates in the ABI 7900HT Sequence Detection System using 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Cycle threshold (C_t) values were obtained for each sample run in three wells, and the averages and standard deviations were calculated. To normalize the *smad2*, *smad3*, and *tgf-β2* samples, the ΔC_t values were calculated by subtracting the C_t value of each sample from the C_t of GAPDH. The $\Delta\Delta C_t$ values were gathered by subtracting the ΔC_t values of each sample from that of the CSV control sample. Relative mRNA expression levels of each gene were then obtained by $2^{-\Delta\Delta Ct}$ calculations. The normalized relative expression levels of each gene are the result of three experiments.

Plasmid construction and luciferase reporter assays

The pGL3–6×SBS reporter vector used in the luciferase assays contains six tandem TGF-β-inducible Smad3-binding sites (SBS). Oligonucleotides containing the 6×SBS binding site with flanking *Kpn*I and *Mlu*I restriction sites were designed as follows: 6×SBS top 5′-CAGCCAGACAAAAAGCCAGACATTTAGCCAGACAGAGCTCAGCCA-GACAAAAAGCCAGACATTTAGCCAGACAA-3′; 6×SBS bottom 5′-CTGTCTGGCTAAATGTCTGGCTTTTTGTCTGGCTGG-TACCGCGTTGTCTGGCTAAATGTCTGGCTTTTTGTCTGGCTGAGCT-3′. By annealing these complementary oligonucleotides, the 6×SBS binding site was created and cloned into the pGL3 reporter vector (Promega). For construction of expression vectors, wild-type and DN *smad3* fragments were excised from the pGEM-T easy vector by digestion with *Eco*RV. The gene fragments were then cloned in-frame into the pRc/RSV expression vector (Invitrogen Life Technologies) digested with *Hind*III and blunted for 5′ overhangs.

Reporter assays were performed as previously described (Majid et al., 2006). Briefly, CEF cultures were seeded at a density of 6×10^5 cells/60 mm tissue culture plate 24 h prior to transfection. DNA transfections were carried out with 0.25 μg pRL-TK, 0.2 μg pGL3–6×SBS reporter vector, 0.5 μg empty pRc/RSV expression vector or one containing the wild-type or DN *smad3* genes. The pBluescript SK+ plasmid was used to maintain a total DNA concentration of 10 μg. The DNA was mixed with 2.5 M $CaCl_2$ and an equal volume of 2× HEPES-buffered saline solution (HBSS). Eight hours later, cells were treated with glycerol shock solution (15% glycerol, 2× HBSS). After an additional 24 h, cells were harvested and luciferase activity measured using the Dual Luciferase Reporter Assay System, according to the manufacturer's instructions (Promega Corporation). To measure luciferase activity in response to TGF-β, cells were treated with 2 ng/ml TGF-β2 (R&D Systems) for 30–45 min prior to harvest at 32 h post-transfection. For experiments in CEFs infected with RCAS retroviruses expressing shRNAs, cells were transfected with 500 ng of the Negative Control

Reporter or Smad-responsive reporter from the Cignal Smad Reporter Kit (SABiosciences, Frederick, MA). Glycerol shock and TGF-β2 treatment prior to harvest was performed as described above. For all assays, luciferase activity was normalized to values of transfection efficiency derived from the pRL-TK vector. The adjusted luciferase activity obtained for cells transfected with the empty pRc/RSV vector was considered baseline activity, and the fold induction was determined by reference to this baseline. For experiments with CEFs infected with retroviruses expressing v-Rel or CSV, the adjusted luciferase activity obtained from CSV-infected cells was considered baseline. For experiments with CEFs infected with RCAS retroviral vectors expressing shRNAs, the luciferase activity from the Smad-responsive reporter for each sample was normalized to that of the Negative Control reporter and then compared against the baseline activity of the empty RCAS vector. P-values were calculated from two-tailed Student's *t*-test comparing luciferase activity of the control cells to other samples.

Soft agar colony assays of cell lines

Plating of v-Rel transformed cells in soft agar has been previously described (Majid et al., 2006). v-Rel-transformed cell lines (160/2, 123/12, and 123/6T) were infected with empty DS vector or DS retroviruses expressing wild-type and DN Smad3 at a multiplicity of infection (MOI) of 7–10 for eight days before viable cells were counted and plated in 0.35% soft agar. The plating media consisted of 0.35% Noble Agar (Becton, Dickinson and Company, Sparks, MD) and DMEM containing 5% FCS, 5% CS, and 1% Antibiotic–Antimycotic reagent. For colony assays with the SB-431542 or SB-525334 TβIR inhibitor (Sigma-Aldrich, St Louis, MO), cells were treated with 3 μM of the inhibitor for 6 h prior to soft agar plating. In addition, 3 μM of the inhibitor or DMSO was added to the plating mix. 160/2 and 123/12 were plated at a concentration of 1.5×10^4 and 1×10^4, respectively while 123/6T and DT40 cells were plated at 5×10^3 cells/plate. Plates were incubated at 37 °C and 8% CO_2, and colonies were scored 7–10 days post-plating. P-values from colony assays with infected v-Rel cell lines were calculated from two-tailed Student's *t*-test comparing the transformation efficiency of cells infected with empty DS vector with those infected with DS expressing WT and DN Smad3. For colony assays performed with cells treated with the inhibitors, P-values were calculated from Student's *t*-test comparing the transformation efficiency of DMSO-treated cells with those treated with the inhibitors.

In vitro transformation assays

Primary splenic lymphocytes were isolated from three-week old White Leghorn chickens, as previously described (Majid et al., 2006). Lymphocytes were purified from spleens by differential gradient centrifugation with Histopaque-1077 (Sigma-Aldrich). Viable cells were counted and resuspended at a density of 2×10^8 cells/ml and infected with REV-TW retroviruses with a minimum titer of 1×10^5 infectious units/ml. Cells (150 μl) were infected at an MOI of 0.01 in a total volume of 3 ml. Infections were allowed to proceed for 24 h after which cells were plated in 0.35% soft agar medium. Experiments with TGF-β2 involved treating cells for 30 min prior to soft agar plating while those with the SB-431542 TβIR inhibitor involved 6 h of pre-treatment. The plating media was the same as described above for colony assays with v-Rel-transformed cells except that 15% FCS was used instead of 5% FCS. In addition, appropriate concentrations of TGF-β2 or TβIR inhibitor were added to the plating mix. For liquid transformation assays, splenic lymphocytes were infected with CSV and the REV-C retrovirus and a day later, cells from each infection underwent eight 1:2 serial dilutions. Dilutions for each infection were treated with 0.5 ng/ml TGF-β2 or carrier. 200 μl of each dilution was then aliquoted into 12 wells in a 96-well plate. For both *in vitro* and liquid transformation assays, plates were kept at 37 °C and 8% CO_2 and colonies scored 10 days later. Liquid transformation efficiency was calculated by multiplying the number of wells showing visible growth for a specific dilution by the reciprocal of that dilution. P-values were calculated from two-tailed Student's *t*-test comparing the transformation efficiency of v-Rel-infected cells treated with carrier solution to those treated with TGF-β2 or TβIR inhibitor.

TGF-β2 Enzyme-linked immunosorbent assays

CEFs and DT40 cells were infected with CSV or with REV-C or REV-TW retroviruses with a minimum titer of 1×10^5 infectious units/ml. For CEF infections, cells were plated at a density of 6×10^5 cells/60 mm tissue culture plate. About 24 h later, CEFs were infected with 4 ml of virus with normalized titers. DT40 infections (5×10^6 cells/infection) were carried out in 5 ml of virus with normalized titers. Over a 10–14 day time course of infection, 0.5 ml of media was harvested from CEFs when they reached confluency and from DT40 cultures when they reached a density of at least 3×10^6 cells/ml and stored at −80 °C. CEFs were passaged at a 1:3 split every 2–3 days whereas DT40 cells were resuspended at a density of 1.5×10^6 cells/ml every 3–4 days. At the end of the time course of infection, ELISAs were performed on the media samples using the Quantikine Human TGF-β2 Immunoassay (R&D Systems), according to the manufacturer's instructions.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.02.002.

Acknowledgments

We thank Juliana Sheely, Radmila Hrdlickova, and Andrew Liss for critical reading of the manuscript and Edward B. Leof for providing the phospho-Smad3 antibody used in early experiments. This study was supported by Public Health Service grants CA33192 and CA098151 from the National Cancer Institute.

References

Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., Wrana, J.L., 1997. TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2–Smad4 complex formation and signaling. J Biol Chem 272, 27678–27685.

Aggarwal, B.B., 2004. Nuclear factor-κB: the enemy within. Cancer Cell 6, 203–208.

Arsura, M., Panta, G.R., Bilyeu, J.D., Cavin, L.G., Sovak, M.A., Oliver, A.A., Factor, V., Heuchel, R., Mercurio, F., Thorgeirsson, S.S., Sonenshein, G.E., 2003. Transient activation of NF-κB through a TAK1/IKK kinase pathway by TGF-β1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene 22, 412–425.

Attisano, L., Wrana, J.L., 1998. Mads and Smads in TGF-β signalling. Curr Opin Cell Biol 10, 188–194.

Bonizzi, G., Karin, M., 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25, 280–288.

Chen, F.E., Ghosh, G., 1999. Regulation of DNA binding by Rel/NF-κB transcription factors: structural views. Oncogene 18, 6845–6852.

Cohen, J., Chen, Z., Lu, S.L., Yang, X.P., Arun, P., Ehsanian, R., Brown, M.S., Lu, H., Yan, B., Diallo, O., Wang, X.J., Van Waes, C., 2009. Attenuated transforming growth factor β signaling promotes nuclear factor-κB activation in head and neck cancer. Cancer Res 69, 3415–3424.

Correia, J.J., Chacko, B.M., Lam, S.S., Lin, K., 2001. Sedimentation studies reveal a direct role of phosphorylation in Smad3:Smad4 homo- and hetero-trimerization. Biochemistry 40, 1473–1482.

Das, R.M., Van Hateren, N.J., Howell, G.R., Farrell, E.R., Bangs, F.K., Porteous, V.C., Manning, E.M., McGrew, M.J., Ohyama, K., Sacco, M.A., Halley, P.A., Sang, H.M., Storey, K.G., Placzek, M., Tickle, C., Nair, V.K., Wilson, S.A., 2006. A robust system for RNA interference in the chicken using a modified microRNA operon. Dev Biol 294, 554–563.

de Caestecker, M.P., Piek, E., Roberts, A.B., 2000. Role of transforming growth factor-β signaling in cancer. J Natl Cancer Inst 92, 1388–1402.

de Jonge, R.R., Garrigue-Antar, L., Vellucci, V.F., Reiss, M., 1997. Frequent inactivation of the transforming growth factor β type II receptor in small-cell lung carcinoma cells. Oncol Res 9, 89–98.

Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425, 577–584.

Gilmore, T.D., 1999. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene 18, 6925–6937.

Go, C., Li, P., Wang, X.J., 1999. Blocking transforming growth factor β signaling in transgenic epidermis accelerates chemical carcinogenesis: a mechanism associated with increased angiogenesis. Cancer Res 59, 2861–2868.

Grygielko, E.T., Martin, W.M., Tweed, C., Thornton, P., Harling, J., Brooks, D.P., Laping, N.J., 2005. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming

growth factor-β type I receptor kinase in puromycin-induced nephritis. J Pharmacol Exp Ther 313, 943–951.
Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H., Kern, S.E., 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271, 350–353.
Halder, S.K., Beauchamp, R.D., Datta, P.K., 2005. A specific inhibitor of TGF-β receptor kinase, SB-431542, as a potent antitumor agent for human cancers. Neoplasia 7, 509–521.
Han, S.U., Kim, H.T., Seong, D.H., Kim, Y.S., Park, Y.S., Bang, Y.J., Yang, H.K., Kim, S.J., 2004. Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. Oncogene 23, 1333–1341.
He, W., Cao, T., Smith, D.A., Myers, T.E., Wang, X.J., 2001. Smads mediate signaling of the TGF-β superfamily in normal keratinocytes but are lost during skin chemical carcinogenesis. Oncogene 20, 471–483.
Henckaerts, E., Langer, J.C., Orenstein, J., Snoeck, H.W., 2004. The positive regulatory effect of TGF-β2 on primitive murine hemopoietic stem and progenitor cells is dependent on age, genetic background, and serum factors. J Immunol 173, 2486–2493.
Hjelmeland, M.D., Hjelmeland, A.B., Sathornsumetee, S., Reese, E.D., Herbstreith, M.H., Laping, N.J., Friedman, H.S., Bigner, D.D., Wang, X.F., Rich, J.N., 2004. SB-431542, a small molecule transforming growth factor-β-receptor antagonist, inhibits human glioma cell line proliferation and motility. Mol Cancer Ther 3, 737–745.
Hrdlickova, R., Nehyba, J., Humphries, E.H., 1994. In vivo evolution of *c-rel* oncogenic potential. J Virol 68, 2371–2382.
Hrdlickova, R., Nehyba, J., Bose Jr., H.R., 1995. Mutations in the DNA-binding and dimerization domains of v-Rel are responsible for altered κB DNA-binding complexes in transformed cells. J Virol 69, 3369–3380.
Hrdlickova, R., Nehyba, J., Bose Jr., H.R., 2001. Interferon regulatory factor 4 contributes to transformation of v-Rel-expressing fibroblasts. Mol Cell Biol 21, 6369–6386.
Inman, G.J., Allday, M.J., 2000. Resistance to TGF-β1 correlates with a reduction of TGF-β type II receptor expression in Burkitt's lymphoma and Epstein–Barr virus-transformed B lymphoblastoid cell lines. J Gen Virol 81, 1567–1578.
Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H., Grunert, S., 2002. Ras and TGF-β cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 156, 299–313.
Javelaud, D., Mauviel, A., 2005. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-β: implications for carcinogenesis. Oncogene 24, 5742–5750.
Kabrun, N., Hodgson, J.W., Doemer, M., Mak, G., Franza Jr., B.R., Enrietto, P.J., 1991. Interaction of the v-Rel protein with an NF-κB DNA binding site. Proc Natl Acad Sci USA 88, 1783–1787.
Kale, V.P., Vaidya, A.A., 2004. Molecular mechanisms behind the dose-dependent differential activation of MAPK pathways induced by transforming growth factor-β1 in hematopoietic cells. Stem Cells Dev 13, 536–547.
Karin, M., Cao, Y., Greten, F.R., Li, Z.W., 2002. NF-κB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2, 301–310.
Karst, M., Gorny, G., Galvin, R.J., Oursler, M.J., 2004. Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-β regulation of osteoclast differentiation. J Cell Physiol 200, 99–106.
Kawabata, M., Inoue, H., Hanyu, A., Imamura, T., Miyazono, K., 1998. Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. EMBO J 17, 4056–4065.
Kawabata, M., Imamura, T., Inoue, H., Hanai, J., Nishihara, A., Hanyu, A., Takase, M., Ishidou, Y., Udagawa, Y., Oeda, E., Goto, D., Yagi, K., Kato, M., Miyazono, K., 1999. Intracellular signaling of the TGF-β superfamily by Smad proteins. Ann NY Acad Sci 886, 73–82.
Ko, Y., Koli, K.M., Banerji, S.S., Li, W., Zborowska, E., Willson, J.K., Brattain, M.G., Arteaga, C.L., 1998. A kinase-defective transforming growth factor-β receptor type II is a dominant-negative regulator for human breast carcinoma MCF-7 cells. Int J Oncol 12, 87–94.
Koli, K.M., Ramsey, T.T., Ko, Y., Dugger, T.C., Brattain, M.G., Arteaga, C.L., 1997. Blockade of transforming growth factor-β signaling does not abrogate antiestrogen-induced growth inhibition of human breast carcinoma cells. J Biol Chem 272, 8296–8302.
Kralova, J., Sheely, J., Liss, A.S., Bose, H.R., 2010. ERK and JNK activation is essential for oncogenic transformation by v-Rel. Oncogene.
Kurokawa, M., Mitani, K., Imai, Y., Ogawa, S., Yazaki, Y., Hirai, H., 1998. The t(3;21) fusion product, AML1/Evi-1, interacts with Smad3 and blocks transforming growth factor-β-mediated growth inhibition of myeloid cells. Blood 92, 4003–4012.
Lange, D., Persson, U., Wollina, U., ten Dijke, P., Castelli, E., Heldin, C.H., Funa, K., 1999. Expression of TGF-β related Smad proteins in human epithelial skin tumors. Int J Oncol 14, 1049–1056.
Liss, A.S., Bose Jr., H.R., 2002. Mutational analysis of the v-Rel dimerization interface reveals a critical role for v-Rel homodimers in transformation. J Virol 76, 4928–4939.
Liss, A.S., Bose, H.R., 2008. Reticuloendotheliosis viruses, In: Mahy, B., Van Regenmortel, M. (Eds.), Encyclopedia of Virology, 3rd ed. , pp. 412–419.
Liu, X., Sun, Y., Constantinescu, S.N., Karam, E., Weinberg, R.A., Lodish, H.F., 1997. Transforming growth factor β-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. Proc Natl Acad Sci USA 94, 10669–10674.
Luque, I., Gelinas, C., 1997. Rel/NF-κB and IκB factors in oncogenesis. Semin Cancer Biol 8, 103–111.
Macias-Silva, M., Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L., Wrana, J.L., 1996. MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell 87, 1215–1224.
Majid, S.M., Liss, A.S., You, M., Bose, H.R., 2006. The suppression of SH3BGRL is important for v-Rel-mediated transformation. Oncogene 25, 756–768.
Margadant, C., Sonnenberg, A., 2010. Integrin-TGF-β crosstalk in fibrosis, cancer and wound healing. EMBO Rep 11, 97–105.
Massague, J., 2008. TGFβ in cancer. Cell 134, 215–230.
Matsuyama, S., Iwadate, M., Kondo, M., Saitoh, M., Hanyu, A., Shimizu, K., Aburatani, H., Mishima, H.K., Imamura, T., Miyazono, K., Miyazawa, K., 2003. SB-431542 and Gleevec inhibit transforming growth factor-β-induced proliferation of human osteosarcoma cells. Cancer Res 63, 7791–7798.
Moustakas, A., Heldin, C.H., 2005. Non-Smad TGF-β signals. J Cell Sci 118, 3573–3584.
Muraoka, R.S., Koh, Y., Roebuck, L.R., Sanders, M.E., Brantley-Sieders, D., Gorska, A.E., Moses, H.L., Arteaga, C.L., 2003. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor β1. Mol Cell Biol 23, 8691–8703.
Nehyba, J., Hrdlickova, R., Bose Jr., H.R., 1997. Differences in κB DNA-binding properties of v-Rel and c-Rel are the result of oncogenic mutations in three distinct functional regions of the Rel protein. Oncogene 14, 2881–2897.
Petersen, M., Pardali, E., van der Horst, G., Cheung, H., van den Hoogen, C., van der Pluijm, G., Ten Dijke, P., 2009. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene 29 (9), 1351–1361.
Poncelet, A.C., de Caestecker, M.P., Schnaper, H.W., 1999. The transforming growth factor-β/SMAD signaling pathway is present and functional in human mesangial cells. Kidney Int 56, 1354–1365.
Rahimi, R.A., Leof, E.B., 2007. TGF-β signaling: a tale of two responses. J Cell Biochem 102, 593–608.
Robetorye, R.S., Bohling, S.D., Morgan, J.W., Fillmore, G.C., Lim, M.S., Elenitoba-Johnson, K.S., 2002. Microarray analysis of B-cell lymphoma cell lines with the t(14;18). J Mol Diagn 4, 123–136.
Sachdev, S., Hannink, M., 1998. Loss of IκBα-mediated control over nuclear import and DNA binding enables oncogenic activation of c-Rel. Mol Cell Biol 18, 5445–5456.
Sood, R., Talwar-Trikha, A., Chakrabarti, S.R., Nucifora, G., 1999. MDS1/EVI1 enhances TGF-β1 signaling and strengthens its growth-inhibitory effect but the leukemia-associated fusion protein AML1/MDS1/EVI1, product of the t(3;21), abrogates growth-inhibition in response to TGF-β1. Leukemia 13, 348–357.
Takaku, K., Miyoshi, H., Matsunaga, A., Oshima, M., Sasaki, N., Taketo, M.M., 1999. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res 59, 6113–6117.
ten Dijke, P., Hill, C.S., 2004. New insights into TGF-β-Smad signalling. Trends Biochem Sci 29, 265–273.
Tzankov, A., Krugmann, J., Fend, F., Fischhofer, M., Greil, R., Dirnhofer, S., 2003. Prognostic significance of CD20 expression in classical Hodgkin lymphoma: a clinicopathological study of 119 cases. Clin Cancer Res 9, 1381–1386.
Verrecchia, F., Chu, M.L., Mauviel, A., 2001. Identification of novel TGF-β /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J Biol Chem 276, 17058–17062.
Wang, S.E., Yu, Y., Criswell, T.L., Debusk, L.M., Lin, P.C., Zent, R., Johnson, D.H., Ren, X., Arteaga, C.L., 2010. Oncogenic mutations regulate tumor microenvironment through induction of growth factors and angiogenic mediators. Oncogene 29, 3335–3348.
White, D.W., Gilmore, T.D., 1993. Temperature-sensitive transforming mutants of the *v-rel* oncogene. J Virol 67, 6876–6881.
Wu, R.Y., Zhang, Y., Feng, X.H., Derynck, R., 1997. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. Mol Cell Biol 17, 2521–2528.
Xu, X., Brodie, S.G., Yang, X., Im, Y.H., Parks, W.T., Chen, L., Zhou, Y.X., Weinstein, M., Kim, S.J., Deng, C.X., 2000. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. Oncogene 19, 1868–1874.
Zaninoni, A., Imperiali, F.G., Pasquini, C., Zanella, A., Barcellini, W., 2003. Cytokine modulation of nuclear factor-κB activity in B-chronic lymphocytic leukemia. Exp Hematol 31, 185–190.
Zhang, Y., Feng, X., We, R., Derynck, R., 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-β response. Nature 383, 168–172.